

Hypoxia causes IL-8 secretion, Charcot Leyden crystal formation, and suppression of corticosteroid-induced apoptosis in human eosinophils

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Short title: Hypoxia and eosinophil function

Key words: Eosinophils; hypoxia; corticosteroids; apoptosis; IL-8

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24 **Abstract**

25 *Background* Inflamed environments are typically hypercellular, rich in pro-inflammatory
26 cytokines, and profoundly hypoxic. While the effects of hypoxia on neutrophil longevity
27 and function have been widely studied, little is known about the consequences of this
28 stimulus on eosinophils.

29 *Objective* We sought to investigate the effects of hypoxia on several key aspects of
30 eosinophil biology; namely secretion, survival, and their sensitivity to
31 glucocorticosteroids (GCS), agents which normally induce eosinophil apoptosis.

32 *Methods* Eosinophils derived from patients with asthma/atopy or healthy controls were
33 incubated under normoxia and hypoxia, with or without glucocorticoids. Activation was
34 measured by flow cytometry, ELISA of cultured supernatants and F-actin staining;
35 apoptosis and efferocytosis by morphology and flow cytometry, and GCS efficacy by
36 apoptosis assays and qPCR.

37 *Results* Hypoxic incubation (3 kPa) caused: (i) stabilisation of HIF-2 α and up-regulation
38 of hypoxia regulated genes including BNIP3 (BCL2/adenovirus E1B 19 kDa protein-
39 interacting protein 3) and GLUT1 (glucose transporter 1), (ii) secretion of pre-formed IL-
40 8, and Charcot Leyden crystal (CLC) formation, that was most evident in eosinophils
41 derived from atopic and asthmatic donors, (iii) enhanced F-actin formation, (iv) marked
42 prolongation of eosinophil lifespan (via a NF- κ B and Class I PI3-kinase-dependent
43 mechanism), and (v) complete abrogation of the normal pro-apoptotic effect of
44 dexamethasone and fluticasone furoate. This latter effect was evident despite
45 preservation of GCS-mediated gene transactivation under hypoxia.

Conclusion and Clinical Relevance These data indicate that hypoxia promotes an eosinophil pro-inflammatory phenotype by enhancing eosinophil secretory function, delaying constitutive apoptosis and importantly, antagonising the normal pro-apoptotic effect of GCS. Since eosinophils typically accumulate at sites that are relatively hypoxic, particularly during periods of inflammation, these findings may have important implications to understanding the behaviour these cells *in vivo*.

Introduction

Eosinophils are innate immune cells involved in allergic inflammation. While recent studies have highlighted certain beneficial effects of eosinophils (e.g. to support muscle regeneration [1], maintain bone marrow plasma cell numbers [2], regulate the biogenesis of beige fat [3] and promote respiratory syncytial virus clearance [4]), most indicate a pathogenic role for these cells in inflammation [5]. The damaging potential of eosinophils has been attributed to their ability to generate and secrete an array of highly histotoxic products, most of which are contained within pre-stored granules; this is achieved either by exocytosis, cytolysis or piecemeal degranulation [6]. These processes permit the selective release of a highly active ‘secretome’ consisting of cationic proteins, pro-inflammatory cytokines, bio-active lipids and reactive oxygen intermediates. In addition, Charcot Leyden crystals (CLCs) are eosinophil-derived bipyramidal structures found in tissues and body fluids of patients suffering from eosinophilic inflammation, typically affecting the airways [7]. While the dominant CLC protein (galectin-10) has now been recognised as a member of the lectin family [8], the processes leading to CLC formation remain poorly understood. Moreover, the CLC protein appears to be highly

69 pro-inflammatory; for example, galectin-10 mRNA is overexpressed in aspirin-induced
70 asthma and CLCs have been shown to damage respiratory epithelium and increase
71 vascular permeability [9].
72
73 Allergic inflammation is thought to delay the capacity of eosinophils to undergo
74 constitutive apoptosis [10], and in animal models, accelerating eosinophil apoptosis
75 promotes the resolution of allergic inflammation [11]. Corticosteroids, working through
76 the glucocorticoid receptor (GR), are highly efficient in suppressing allergic
77 inflammation, in part through their capacity to suppress degranulation and secretory
78 responses and potentially also through their ability to drive eosinophil apoptosis [12][13].
79 However, despite the exquisite sensitivity of eosinophils to the pro-apoptotic effects of
80 GR agonists *in vitro*, a significant subset of patients with eosinophilic inflammation
81 exhibit glucocorticoid-resistant disease; such individuals present a major therapeutic
82 conundrum and utilise disproportionate health care resources [14]. Of note, much of
83 the experimental work undertaken to define the biology of eosinophils has been
84 conducted under ambient oxygen concentrations, typically 21 kPa. This relatively
85 'hyperoxic' state may have little relevance to the physiological PO_2 these cells encounter
86 *in vivo*, with both sterile and non-sterile inflammation able to reduce the level of tissue
87 oxygenation still further, often to PO_2 values below 1 kPa; this predicates the need for
88 myeloid cells to operate efficiently under hypoxic conditions [15][16][17].

89 We have shown that neutrophils express the oxygen sensing prolyl hydroxylase enzymes
90 PHD1-3 and the transcriptional factors HIF-1 α and HIF-2 α , and although well adapted to
91 survive under hypoxia, are extremely sensitive to the ambient PO_2 [15]. Hence, a drop in

oxygen levels to 3 KPa (which equates to physiological oxygen tensions in the skin [18], gut [19], and bone marrow [20]) causes a marked inhibition of neutrophil NADPH oxidase-dependent ROS generation and bacterial killing [21]. Hypoxia also impairs spontaneous neutrophil apoptosis, the latter through a HIF-1 α - and NF- κ B-dependent pathway [15].

In contrast, eosinophil responses under hypoxia have been far less studied. HIF-1 α and HIF-2 α are both expressed in murine eosinophils and appear to regulate eosinophil chemotaxis [22] and *in vitro*, hypoxia has been reported to up-regulate the inhibitory receptor CD300a, enhance eosinophil viability, and cause a small increase in basal IL-8 and VEGF release [23][24]. However, the effects of hypoxia on the pro-apoptotic and anti-inflammatory effects of corticosteroids on eosinophils are unknown. This question has important biological relevance, not only because of the hypoxic environment commonly encountered by eosinophils *in vivo*, but because of reports in other cell types that hypoxia can induce a state of glucocorticoid resistance [25].

Using ultra-pure human blood eosinophils, we now show that hypoxia is a potent stimulus of spontaneous and agonist-stimulated IL-8 release, an effect which is most evident in cells purified from atopic and asthmatic donors. We also report for the first time that culture of human eosinophils results in overt CLC formation only when cells are purified from atopic donors and, perhaps more critically, when these cultures are performed under hypoxic conditions (PO_2 3 kPa). In addition, we demonstrate that hypoxia antagonises the normal pro-apoptotic effect of dexamethasone and as a consequence reduces the extent of efferocytosis by monocyte-derived macrophages.

115 Mechanistically, the capacity of hypoxia to inhibit eosinophil apoptosis appears to relate
116 to the ability of this stimulus to ‘out-compete’ the normal pro-apoptotic effect of
117 corticosteroids, as GR-mediated nuclear signalling is preserved under hypoxia [26].
118 These studies illustrate the significant effects of physiologically and pathologically
119 relevant levels of hypoxia on eosinophil function, and the capacity of hypoxia to
120 attenuate one of the major anti-inflammatory effects of corticosteroids.
121

Methods

These studies were approved by the Cambridge Research Ethics Committee, UK (06/Q0108/281); written informed consent was obtained from all participants.

Isolation of human peripheral blood neutrophils and eosinophils

Human peripheral blood neutrophils were purified from healthy donors using dextran sedimentation and discontinuous plasma-Percoll gradients as detailed [27]. Human eosinophils were isolated from healthy volunteers, mildly atopic donors (with appropriate history and a positive skin prick test to one or more aeroallergens) and individuals with asthma (physician-diagnosed on Step 1 or 2 BTS Guideline treatment), using HetaSep™ hetastarch sedimentation and Robosep® and EasySep® Human Eosinophil Enrichment Kits (Stem Cell Technologies, Manchester, UK), according to manufacturers' instructions. Cell purities (assessed by cytopsin preparations stained with Diff-Quick™) were >95% for neutrophils and >99% for eosinophils (Fig. 1A). Both of these isolation methods have been demonstrated by our group to induce minimal cell priming/activation as judged in neutrophils by lack of basal shape change or oxidative burst to fMLP [28] and in eosinophils by unperturbed cell surface expression of CD69, CD44, CD81 and CD66b, shape change, EM-assessed granule morphology, and eosinophil-derived neurotoxin (EDN) release [29].

Hypoxic culture of eosinophils and neutrophils

Purified eosinophils were re-suspended at $1-2 \times 10^6$ cells/ml in RPMI supplemented with 10% (v/v) autologous serum, 100 U/ml streptomycin and 100 U/ml penicillin. Neutrophils

were re-suspended at 5×10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) autologous serum, 100 U/ml streptomycin and 100 U/ml penicillin or Dulbecco's phosphate buffered saline (PBS) containing CaCl_2 and MgCl_2 (PBS+). Apoptosis assays were undertaken in a final volume of 150 μl in flat-bottomed 96-well (ultra-low attachment) Costar™ plates.

Normoxic incubations utilised media equilibrated in a humidified 5% CO_2 /air incubator (representing 19-21 kPa oxygen) whereas a hypoxic environment (typically an atmospheric oxygen concentration of 0.8%, giving a media PO_2 of 2.8 ± 0.1 KPa, (n) = 20, with PCO_2 and pH values matched to the values under normoxic conditions) was achieved by culturing in a Ruskinn *Invivo* 400 hypoxic incubator. All media were allowed to equilibrate for 3 hr prior to use and hypoxia confirmed using an ABL5 blood gas analyser (Radiometer, Denmark). CO_2 settings were titrated to ensure maintenance of a physiological pH and varied according to the buffering system. The induction of hypoxia at a cellular level was confirmed by showing stabilisation of eosinophil HIF-2 α using anti-HIF-2 α (Novus Biologics, UK) (Fig. 1B). Despite repeated attempts, HIF-1 α could not be reliably detected by Western blot in human eosinophils.

Western blot analysis

Following culture of 1-5 million eosinophils/well in ultra-low attachment 6-well plates for 6 hr under normoxia and hypoxia, the supernatants were removed and cells were lysed with 100 μl of radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium

deoxycholate, 0.1% SDS (sodium dodecyl sulphate) and 50 mM Tris, pH 8.0 containing cOmplete™, EDTA-free Protease Inhibitor Cocktail Tablets, Roche). The plates were snap-frozen using dry ice in industrial methylated spirit (IMS) and stored at -80°C until required, then later scraped and the lysates sonicated and analysed for protein content. Freshly isolated cells were re-suspended in the appropriate supplemented medium at 1-5 million eosinophils/tube and pelleted at 256 g for 5 min at 4°C. Pellets were also re-suspended in 100 µl of RIPA buffer before being snap-frozen. Samples were prepared by mixing with LDS sample buffer (4X) and heated to 70°C (for HIF1/2α detection on Tris-acetate gels) for 10 min and allowed to cool to RT prior to loading and subsequent SDS-PAGE analysis using Tris-acetate 3-15% gels. Membranes were probed for HIF stabilisation using anti-HIF-1α anti-HIF-2α antibodies (Novus Biologics, UK) using the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech) and normalised against p38 protein, as previously described [30].

Assessment of apoptosis

Eosinophil and neutrophil apoptosis was assessed using cell morphology and Annexin-V/PI flow cytometry as described [31]. Since acidic microenvironments have been shown to enhance the viability of eosinophils, the tissue culture media contained 25 mM Hepes and the pH was monitored throughout the experiment [32]. Inhibitors and compounds used to investigate the effects of hypoxia on eosinophil lifespan and function included: NF-κB inhibitor GSK657311A, Class 1A PI3-kinase inhibitor 987740A, CXCR2 antagonist SB-3322357 and the GR modulator GRT10 (all gifted from Dr Stuart

Farrow, GSK); a pan-PI3K inhibitor LY294002 (Calbiochem, Nottingham, UK) and JNK inhibitor SP600125 (Sigma, UK).

Assessment of eosinophil activation

(i) Quantification of CLC formation

CLC formation was assessed by examining Diff-Kwik™ stained cytopins; each treatment was scored for the number of CLCs formed across 5 random fields of view (AU Arbitrary Units; 1 ≤ 25 CLCs [per 5 high power fields (hpfs)]; 2 = 25-50 CLCs [per 5 hpfs]; 3 = 50+ CLCs [per 5 hpfs]).

(ii) Actin polymerisation

Eosinophils were re-suspended at 1×10^6 /ml in PBS without cations (PBS-) and incubated in normoxic and hypoxic PBS- and left for 1 hr prior to stimulation. Cells were then stimulated with fMLP (100 nM), eotaxin (100 nM) or vehicle for the time-points indicated and fixed in 4% formaldehyde. After 1 hr, 100 µl NBD-buffer (NBD-phalloidin in 1.5 ml of absolute methanol, 37% formaldehyde, PBS-, 0.2 mg/ml lysophosphatidylcholine) was added and the cells incubated in the dark for a further hour. Cells were analysed on a Fortessa (BD) flow cytometer using excitation with the 488 nm laser and emission measured at 525 nm (green fluorescence/FL1) [33].

(iii) Analysis of CD69 cell surface expression

Freshly isolated eosinophils were suspended in supplemented RPMI at 2×10^6 cells/ml and incubated in either a normoxic or hypoxic environment. Cells were washed in FACS buffer (PBS-, 2 mM EDTA, 0.5% BSA and 0.1% sodium azide) and re-suspended in 100 µl FACS buffer containing 2.5 µl CD69-FITC conjugated antibody or IgG isotype control

(2.5 µl FITC-mouse IgG1κ). The samples were incubated on ice for 30 min in the dark, washed and re-suspended in 500 µl FACS buffer prior to analysis.

Macrophage phagocytosis assays

Phagocytosis was measured by both light microscopy and flow cytometry. Human monocytes isolated over discontinuous plasma-Percoll gradients were cultured in 24-well tissue culture plates for 7 days in RPMI with 100 ng/ml M-CSF to yield monocyte-derived macrophages (MDMφ). For light microscopy assessment, 2×10^5 MDMφ in RPMI were incubated with 6×10^5 'bait' cells (human neutrophils or eosinophils aged for 20-24 hours *in vitro*) for 1 hour (37°C, 5% CO₂) in a normoxic (21%) or hypoxic environment (0.8%), and then fixed with 2.5% glutaraldehyde. The cells were stained for myeloperoxidase (MPO) with 0.1 mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxide in PBS (MDMφ are MPO-negative) [34]. The percentage of macrophages that had ingested one or more apoptotic granulocyte was quantified by examining a minimum of 300 cells in duplicate wells. To confirm that the apoptotic neutrophils or eosinophils had been internalised, trypsinised cells were cytospun, stained with Diff-Kwik™, and examined under oil immersion.

For flow cytometric quantification of phagocytosis, MDMφ (2×10^5 /ml/200 µl/well) were prepared as above. Normoxic or hypoxic eosinophils (1×10^7 /ml in RPMI without serum) were stained with CMFDA cell tracker dye (1 µl/ 10^7 cells) for 15 min at 37°C. The labelled cells were washed in RPMI and re-suspended at 3×10^6 /ml in serum-free RPMI. MDMφ (6×10^5 /well) were washed and co-incubated with 200 µl of CellTracker Green

CMFDA-labelled apoptotic eosinophils. Following co-incubation for 1 hr at 37°C under a normoxic environment or hypoxic environment, media was replaced with 0.5 ml trypsin-EDTA for 15 min at 37°C and 15 min at 4°C. Vigorous pipetting was performed to ensure detachment of all adherent cells and the extent of MDM ϕ phagocytosis assessed by flow cytometry. MDM ϕ were identified and gated according to their forward and side scatter characteristics; MDM ϕ that had ingested apoptotic eosinophils showed an increase in green fluorescence, becoming FL-1 positive [34].

Cytokine and growth factor ELISAs and quantification of EDN release

Eosinophils (10^6 /ml) were incubated under normoxic or hypoxic conditions for 12 hr and supernatants collected, pooled (2,000 g, 6 min) and stored at -80°C. Cytokines including IL-5, IL-6, IL-8, IL-10 and GM-CSF release were measured by ELISA using 96-well Microton® plates according to the manufacturer's instructions (Qiagen, Crawley, UK). Biotinylated secondary polyclonal antibody was measured using streptavidin conjugated alkaline phosphatase, the plates developed with *p*-nitrophenylphosphate and read at λ 405 nm using a Bio-Rad 550 micro-plate reader. Additional confirmation of cytokine release from eosinophils was undertaken using Qiagen Multi-Analyte ELISArray plates used in accordance to manufacturers' guidelines. EDN release was measured by ELISA, according to manufacturer's guidelines (Immundiagnostik-AG, Bensheim, Germany; lower limit of detection 0.164 ng/ml). Data were analysed using Microsoft Plate Manager (MPM) 1.57 software.

Neutrophil chemotaxis assays

Supernatants from normoxic or hypoxic eosinophils (or IL-8, 100 ng/ml) were placed underneath a NeuroProbe ChemoTx® disposable 96-well filter; freshly isolated neutrophils (5×10^6 cells/ml in IMDM plus 0.1% autologous serum) were added on top of the filter and incubated for 90 min at 37°C in a humidified normoxic incubator. The suspension from each bottom well was collected and the wells washed twice with warm EDTA/trypsin. Cell migration was assessed by haemocytometer cell counting.

Measurement of dexamethasone- and hypoxia-regulated gene expression

For RNA isolation, granulocytes were cultured in 6-well plates at 2×10^7 per well (neutrophils) or $1-5 \times 10^6$ per well (eosinophils), harvested, and the cell pellets re-suspended in 1 ml TRIZOL® (Invitrogen); following chloroform extraction and isopropanol precipitation the RNA pellet was washed x2 with 1 ml ice-cold 70% (v/v) ethanol, air dried, re-suspended in 100 µl of nuclease free water (Promega), and a RNA clean-up and DNase digest performed using RNeasy micro-column kit (Qiagen). A high capacity cDNA kit (Applied Biosystems) was used to generate cDNA using 1 µg of total RNA using the following program settings: 25°C for 10 min, 37°C for 2 hr, 85°C for 5 min and 4°C on hold; RNA preparations were stored at -80°C.

Changes in gene expression were assessed by qPCR using SYBR® Green Jumpstart™ Taq Readymix™ (Sigma), Rox reference dye (Invitrogen) and primers obtained from Qiagen (Suppl. Table 1). The reactions were performed on a StepOnePlus™ (Applied Biosystems) real-time PCR machine or a 384-well 7900HT fast real time PCR machine (Applied Biosystems). Primer efficiency was optimised to obtain the following PCR

settings: 2 min at 95°C for Taq polymerase activation, 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C. Cycle threshold (Ct) values from control and experimental sample sets were normalised to appropriate housekeeping genes (beta-2-microglobulin/YMHAZ/18s) ($\Delta\Delta C_t = \Delta C_t$, sample – ΔC_t) and the relative change in target gene expression (fold change) analysed using the $2^{-\Delta\Delta C_t}$ method [35].

Data Analysis

The results are reported as the mean \pm SD or SE of (n) independent donor experiments with each treatment performed in triplicate for neutrophils and duplicate for eosinophils unless otherwise stated. Data were analysed using the GraphPad Prism statistical analysis package. Paired t-tests were used to compare the means from two groups when samples were obtained from the same donor and were of Gaussian distribution. For the comparison of three or more groups, one-way ANOVA with a post-Tukey's or Dunnett's test was performed or two-way ANOVA was used when more than one variable was assessed, with a post-Tukey's or Dunnett's test for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results

Hypoxia stimulates basal and agonist-mediated IL-8 release from human eosinophils

To determine whether hypoxia influences the release of inflammatory cytokines, human eosinophils were isolated and the supernatants collected following 12 hr of normoxia or hypoxia culture. From the panel of chemokines and cytokines examined, enhanced IL-8 levels and MIP-1 β were observed in all subjects following hypoxic culture (Fig. 1C and Fig. S1). Furthermore, the extent of IL-8 release correlated with the clinical diagnosis, with eosinophils from individuals with atopy and asthma having the highest levels of IL-8 secretion under hypoxia (Fig. 1C, values for asthmatic subjects are within the dashed box).

To investigate whether the enhanced release of IL-8 under hypoxia involved enhanced transcription, eosinophils were cultured under normoxia or hypoxia and the level of IL-8 mRNA assessed (Fig. 1D). Hypoxia led to stabilisation of eosinophil HIF-2 α protein (Figure 1B) and up-regulation of HIF-1 α -regulated transcripts BNIP3 and GLUT1 mRNA, demonstrating activation of both HIF-1 α and HIF-2 α -dependent signalling pathways; in contrast however, hypoxia had no effect on IL-8 mRNA levels (Fig. 1D). Hence the increased IL-8 release evident under hypoxia may reflect enhanced release of pre-formed IL-8 as opposed to *de novo* biosynthesis; attempts to explore this further using transcriptional and protein synthesis inhibitors were thwarted by the extreme sensitivity of eosinophils to agents such as cyclohexamide, which induces a profound pro-apoptotic response, even at concentrations of 0.1 μ g/ml.

To determine if the IL-8 released under hypoxia was biologically active, eosinophil-derived supernatants were assessed in a neutrophil chemotaxis assay using rhIL-8 as a positive control. As shown in Fig. 1E, supernatants derived from eosinophils (from both healthy and atopic donors) cultured *ex vivo* under hypoxia induced neutrophil chemotaxis to the same extent as a pre-determined optimal concentration of rhIL-8, and this was inhibited by the CXCR2 antagonist SB332235Z. A similar trend was observed for supernatants derived from eosinophils incubated under normoxia (Fig. 1E). These data suggest that particularly within the setting of hypoxic inflammation, eosinophils may be a significant source of IL-8, capable of promoting neutrophil influx.

Effects of hypoxia on eosinophil degranulation and polarisation

Piecemeal degranulation is a unique eosinophil secretory mechanism, which results in a selective liberation of cytokines and chemokines and leads to a stimulus-specific eosinophil secretory profile [27][36]. In view of the effect of hypoxia on eosinophil IL-8 release, we investigated additional biologically relevant secretory products and the activation status of eosinophils following hypoxic incubation.

Charcot Leyden crystals are a marker of eosinophil involvement in inflammatory reactions, and persist after eosinophil death/clearance. However, their genesis is poorly understood. Eosinophils derived from healthy volunteers cultured under either normoxia or hypoxia failed to show any CLC formation; in contrast, eosinophils derived from atopic or asthmatic donors cultured under hypoxia for over 24 hours showed prominent CLC formation (Fig. 2A-B), which was not observed under normoxic conditions. Co-

incubation of eosinophils with dexamethasone (100 nM) did not affect basal or hypoxia-induced CLC formation (Fig. 2B). These data support the view that eosinophils from atopic donors differ from healthy donor cells and suggest that hypoxia is an important and previously unrecognised factor in CLC formation.

The conversion of monomeric to filamentous actin (F-actin) is a central process underlying granulocyte motility and exocytosis. Eosinophils cultured under hypoxia exhibited a greater degree of eotaxin-induced actin polymerisation compared to cells stimulated under normoxia (Fig. 2C and Fig. S2). Although the fold increase in total cell F-actin content is small, localised actin polymerisation in discrete areas of the cell is essential for vesicle fusion, hence small focal increases may have profound biological relevance. Given this, we predicted that hypoxia might impact globally on eosinophil secretion. However, as shown in Fig. 2D the extent of IL-5-induced eosinophil-derived neurotoxin (EDN) release was actually attenuated under hypoxia; this inhibitory effect was seen in eosinophils derived from both healthy volunteers and asthmatic subjects (Fig. 2D and data not shown). Likewise, hypoxia had no effect on basal or GM-CSF-stimulated CD69 expression (data not shown), which is also stored in eosinophil granules and upregulated on the eosinophil surface following cytokine stimulation or whole lung antigen challenge [37]. Together these data demonstrate that hypoxia has a nuanced effect on eosinophil secretion, specifically increasing IL-8 release and CLC formation from eosinophils derived from asthmatic/atopic but limiting the liberation of EDN.

Hypoxia promotes eosinophil survival and reduces phagocytic uptake by macrophages

Although debated, impaired eosinophil apoptosis and defective phagocytic clearance (efferocytosis) has been proposed to contribute to the persistence of allergic inflammation. To determine the effects of hypoxia on eosinophil lifespan, these cells were cultured in normoxia or hypoxia in the absence or presence of IL-5, a known pro-survival stimulus. The percentage of apoptotic eosinophils measured by flow cytometry (Fig. 3A) and morphology (Fig. 3B) at 24 hours was markedly reduced by hypoxia, to a level comparable to that seen with IL-5. Analysis of the supernatants derived from hypoxia-cultured eosinophils showed no evidence of IL-5 or GM-CSF release suggesting that this was not due to an autocrine effect of these agents (data not shown).

To determine whether the pro-survival effect of hypoxia on eosinophils might impair eosinophil clearance, the degree of eosinophil efferocytosis by M-CSF-differentiated MDM ϕ was assessed. As shown in Fig. 3C, MDM ϕ uptake of eosinophils was markedly reduced when the eosinophils presented had been pre-incubated under hypoxia rather than normoxia during the previous 24 hours. A subset of MDM ϕ s were also placed under hypoxia for the duration of the efferocytosis assay but this had no effect on their capacity to ingest apoptotic eosinophils (Fig. 3C). This contrasts to the ability of hypoxia or hypoxia mimetics to blunt the capacity of MDM ϕ s to efferocytose apoptotic neutrophils (see Fig. S3). To confirm that we were examining true efferocytosis, a subset of MDM ϕ s were trypsinised at the end of the incubation period and examined by light microscopy, which clearly showed apoptotic cells contained within MDM ϕ (data not shown). These data indicate that hypoxia impairs eosinophil apoptosis and thus clearance *in vitro*.

Hypoxic-mediated eosinophil survival is regulated by NF- κ B and PI3-kinase

To explore the role of NF- κ B in conferring the pro-survival effect of hypoxia on eosinophils (as previously demonstrated in neutrophils [15]), eosinophils were incubated with the selective IKK α inhibitor GSK657311A (1-30 μ M; GSK, Stevenage, UK); comparative studies were also undertaken with the PI3-kinase inhibitor LY294002 (Calbiochem, Nottingham, UK), the more selective PI3-kinase Class I inhibitor 987740A (GSK, Stevenage, UK) and the JNK inhibitor SP600125 (Sigma-Aldrich, Dorset, UK). Unlike the effects seen in neutrophils, the IKK α inhibitor GSK657311A (Fig. 4A) and PI3-kinase inhibitor 987740A (from GSK, Stevenage, UK) (Fig. 4B), both caused a concentration-dependent induction of constitutive apoptosis in eosinophils even under normoxic conditions, suggesting that both signalling pathways play a tonic survival role in these cells [15][38]. Despite this, hypoxic eosinophil survival was attenuated by GSK657311A (at 30 μ M), LY294002 (at 10 μ M) and 987740A (at 10 μ M), suggesting a role for both NF- κ B and PI3-kinase signalling in this response (Fig. 4A-B). JNK pathway inhibition with SP600125 had no effect on eosinophil apoptosis under either normoxia or hypoxia (Fig. 4B).

Hypoxia attenuates dexamethasone-induced eosinophil apoptosis

Having established that hypoxia has a selective effect on eosinophil secretion and induces a marked survival response, we wished to assess whether hypoxia affected the capacity of corticosteroids to induce eosinophil apoptosis. Given that low oxygen tensions have been associated with reduced GR expression and function in other cells [39], we predicted that GR-induced eosinophil apoptosis might be impaired under hypoxia. As shown in Fig. 5A-

D, precisely this effect was seen, with hypoxia causing a profound suppression of the normal concentration-dependent, pro-apoptotic effect of dexamethasone. This was evident using either morphology or AnV/PI-binding to quantify apoptosis, was additive to the survival effect of IL-5, and was observed for up to 72 hours of culture (Fig. 5D); these data also confirmed the ability of the GR antagonist RU486 to inhibit the pro-apoptotic effect of dexamethasone (Fig. 5A). Remarkably, the combination of IL-5 and hypoxia resulted in over 80% eosinophil survival even after 120 hours in culture compared to 100% apoptosis/necrosis of eosinophils under normoxic conditions (Fig. 5D). Hypoxia also blocked the capacity of other high potency GCs (e.g. fluticasone fumarate) to induce eosinophil apoptosis (Fig. 5B). Of note, the hypoxic inhibition of steroid-induced eosinophil apoptosis could be fully recapitulated by the hypoxic mimetics DFO and DMOG (Fig. 5E).

Mechanism of hypoxia-mediated inhibition of dexamethasone-induced eosinophil-apoptosis

From the above experiments we hypothesised that hypoxia might affect the expression or function of GR in eosinophils. Surprisingly however, dexamethasone mediated up-regulation of *Gilz*, a process that is fully GR-dependent [40] and was entirely preserved under hypoxia (Fig. 6A). In these experiments *Glut1* expression was used as a positive control for hypoxia whilst *Ikb* expression was used as a negative control. This indicates that an intact GR-GRE axis is maintained in these cells and that hypoxia operates in a parallel but dominant manner to suppress steroid-induced eosinophil apoptosis.

Finally, to address whether the pro-apoptotic effect of corticosteroids in eosinophils is mediated via GR-transrepression or GR-transactivation, we examined the effects of a newly described GR modulator (GRT10), which has been reported to display selective GR-transrepressive effects [41]. Under normoxia, GRT10 also caused a concentration-dependent increase in eosinophil apoptosis (Fig. 6B, EC₅₀ 2 nM) (and suppressed neutrophil apoptosis; see Fig. S4a), supporting the view that the pro-apoptotic capacity of GC may be mediated via a GR-transrepressive effect. However, GRT10 was unable to promote eosinophil apoptosis under hypoxia, at any of the concentrations tested, suggesting either that hypoxia renders eosinophils insensitive to the pro-apoptotic effects of GRT10, or like dexamethasone, that the marked hypoxic pro-survival effect is sufficient to override any pro-apoptotic signalling.

Discussion

Inflammatory sites, including the airway wall [42][43], are often profoundly hypoxic. This results from a combination of vascular damage, the build-up of inflammatory debris, enhanced cellular metabolism, and increased oxygen extraction due to activation of NOX2 [44]. In this study we aimed to define the effects of hypoxia on eosinophil lifespan and function, and glucocorticoid sensitivity. We confirm that hypoxia is a potent pro-survival stimulus for eosinophils as well as for neutrophils. We also show for the first time that hypoxia reduces the ability of glucocorticoids to induce eosinophil apoptosis, inducing a state of apparent or ‘quasi’-glucocorticoid resistance. Furthermore, hypoxic culture of eosinophils promotes the release of IL-8 to levels capable of inducing

neutrophil chemotaxis and, in cells from atopic donors, supports the formation of CLC. These data indicate that hypoxia can augment a number of potentially detrimental eosinophil functions and promote neutrophil influx.

Hypoxia-induced IL-8 secretion has been reported in cancer [45], endothelial cells [46], epithelial cells and pulmonary fibroblasts [47] as well as macrophages [48]. Consensus sequences for multiple transcription factors (including AP-1, NF- κ B and HIF-1 α) are present in the IL-8 promoter region, enabling context- and cell-dependent IL-8 expression [49] [45]. In agreement with previous results [24], we show that hypoxia induces release of IL-8 from eosinophils; importantly, we also demonstrate that this is more pronounced in cells from atopic and asthmatic donors and is sufficient to promote neutrophil chemotaxis. *In vivo* studies support the potential clinico-pathological relevance of these findings. In mice, Baek *et al.* found that combined allergen and hypoxia challenge resulted in a 27-fold increase in the accumulation of peri-bronchial neutrophils that correlated with the release of KC (a functional homologue of IL-8) from peri-bronchial cells, including eosinophils [50]. IL-8 has been detected in airway secretions of patients with acute severe asthma, contributing to neutrophil recruitment in this setting (e.g. [51][52]); indeed, IL-8 was reported to be the only cytokine in BALF which differentiated controlled from uncontrolled asthma and correlated inversely with FEV₁ [52]. Low oxygen tensions do not promote indiscriminate degranulation; indeed, EDN release was actually inhibited by hypoxia. Selective mobilisation of pre-formed cytokines from eosinophil granule stores by ‘piecemeal degranulation’, a process which involves the trafficking of small vesicles directed by SNAP/SNARE interactions, has

481 been observed previously, although not in response to hypoxia; for example, eotaxin can
482 induce the rapid and selective secretion of IL-4 from eosinophils [53], whilst IFN γ
483 promotes IL-3 and RANTES release [54][55].

484
485 We report for the first time that *in vitro* culture of human eosinophils results in overt CLC
486 formation; intriguingly this was limited to cells purified from atopic donors cultured.
487 These findings align with earlier reports of CLC formation at sites now recognised to be
488 profoundly hypoxic, for example, within inflamed rheumatoid joints and large
489 carcinomas [56] [57]. Given that reduced oxygen tensions have been measured at
490 inflammatory foci and that hypoxia has been shown to regulate galectin-10 expression, it
491 would be tempting to speculate that hypoxia in this instance predicates CLC formation,
492 contributing to local tissue injury [9].

493
494 The pro-survival effect of hypoxia on neutrophils was first described by our group some
495 years ago [58], however the effects of hypoxia on eosinophil lifespan have been little
496 studied. Hypoxia has been shown to increase eosinophil viability, at least in part by the
497 induction of the anti-apoptotic protein Bcl-XL [24]. We confirm that hypoxia is a potent
498 pro-survival stimulus for eosinophils, and demonstrate this hypoxic survival is further
499 augmented by cytokines. Cells recruited to inflammatory sites *in vivo* will undoubtedly
500 experience hypoxia in combination with exposure to pro-inflammatory mediators; in *in*
501 *vitro* experiments designed to re-capitulate this environment, we found that $\geq 80\%$ of
502 eosinophils cultured in the presence of both IL-5 and hypoxia were still fully viable after
503 5 days. Conditions such as asthma and nasal polyposis are characterised by elevated

504 levels of IL-5 and tissue hypoxia, particularly in severe disease [59][60]. Thus, we
505 speculate that the combination of inflammation and hypoxia may exacerbate disease and
506 delay resolution in this and other settings. The physiological relevance of apoptosis to
507 eosinophil clearance *in vivo* remains controversial. While apoptotic eosinophils are
508 readily detected in the sputum of patients with asthma, particularly in the steroid-induced
509 resolution phase, detailed biopsy studies have offered little evidence for this event in the
510 airway wall [61]. Whether hypoxia operates to suppress constitutive apoptosis of
511 eosinophils within microenvironments such as inflamed airways, with re-oxygenation on
512 exposure to oxygen in the airway lumen releasing these constraints is unknown, but
513 would be consistent with mouse data where hypoxia increases peri-bronchial eosinophilia
514 post OVA-challenge [50].

515
516 Glucocorticoids have dichotomous effects on granulocyte lifespan, promoting neutrophil
517 survival yet inducing eosinophil apoptosis [62]. Although corticosteroids are used to
518 modulate inflammatory cell function within environments that may be profoundly
519 hypoxic, few studies have examined the impact of hypoxia on the effects of these agents.
520 Our observation that the effects of glucocorticoids on granulocyte lifespan are
521 significantly attenuated under hypoxia initially suggested that hypoxia may render these
522 cells steroid-insensitive. However, qPCR analysis demonstrated that eosinophils
523 remained intrinsically sensitive to the effects of steroids under hypoxia with
524 dexamethasone retaining the capacity to transactivate genes such as *Gilz* (Fig. 6).
525 Moreover, in preliminary experiments dexamethasone also appears to maintain its
526 transrepressional capacity under hypoxia, causing a reduction (albeit non-significant) in

IL-8 release from eosinophils (Fig. S5). Although in preliminary experiments GRT10 still appeared capable of enhancing some expression of Gilz (regarded as a classically transactivated gene) in eosinophils (Fig. S4 B), similar effects have been seen using other apparently selective glucocorticoid receptor modulators [13], and may suggest an effect mediated by a non-classical glucocorticoid responsive element. Thus, hypoxia appears to render granulocytes ‘quasi’ rather than truly steroid-resistant; we speculate that the reason the pro-apoptotic effects of glucocorticoids are no longer evident under hypoxia reflects the overwhelming pro-survival effect induced by hypoxia i.e. a simple competitive process between pro-apoptotic corticosteroid-driven pathways and an anti-apoptotic hypoxia-regulated mechanisms; under the experimental conditions used, that the hypoxia-induced survival effect dominates, analogous to the situation previously reported in neutrophils treated with the cytokine GM-CSF [26].

The physiological and pathological environments encountered by eosinophils *in vivo* are either known or predicted to be hypoxic compared to arterial or venous blood. The data presented here underscore the sensitivity of eosinophils to ambient oxygen concentration and the significant effects of hypoxia on eosinophil function. These effects were most marked with respect to spontaneous and agonist-stimulated IL-8 and CLC generation, the survival of these cells during *in vitro* culture, and the attenuation of steroid-induced apoptosis. While this paper does not assess the relevance of these findings to the *in vivo* situation, the ‘hypoxic’ PO_2 conditions we used are well within the range of tissue oxygen values recorded, especially under disease conditions.

550 There has been considerable interest and debate regarding the observation that many
551 eosinophil-targeted therapies appear to be more effective in reducing circulating
552 eosinophil numbers compared to their capacity to reduce eosinophil numbers in tissues.
553 One proposed mechanism in the setting of anti-IL-5 therapies is the reduction in IL-5 α
554 receptor expression in bronchoalveolar lavage eosinophils, making these cells far less IL-
555 5-dependent [63]. Our current findings may however provide an additional explanation
556 for the enhanced eosinophil survival seen in the inflamed and hypoxic airway wall and
557 the seeming resistance of these cells to the normal pro-apoptotic effect of corticosteroids.

Figure Legends

Figure 1

Hypoxia promotes stabilization of HIF-2 α and the release of IL-8 from eosinophils.

(a) Human eosinophils were purified from mixed leukocytes using a Robosep® negative selection strategy. (b) Representative Western blot of HIF-2 α and p38 expression immediately after isolation (0 hrs) or following 6 hrs culture of eosinophils under normoxia (N) or hypoxia (H). Data represent mean arbitrary units (\pm SEM) of Western blots analysed by ImageJ from (n) = 4 independent experiments with the HIF-2 α signal normalized to the p38 band. (c) Eosinophils from healthy controls (n=8) or atopic donors (n=12) were cultured for 12 hrs under N or H and IL-8 release assessed. (d) IL-8 mRNA levels from eosinophils cultured for 6 hrs under N or H, normalized to the housekeeping control beta-2 microglobulin. Expression of *Glut1* and *BNIP3* both served as positive controls for HIF-regulated transcripts. Data represent the mean \pm SEM of (n) = 3 independent experiments. (e) Neutrophils were pre-incubated with or without the CXCR2 antagonist SB332235Z (100 nM) for 30 min and the degree of chemotaxis towards eosinophil supernatants (derived from healthy controls following 12 hrs N or H (n=5)) or IL-8 (100 ng/ml; n=3) assessed. Data represent the mean \pm SEM number of migrated neutrophils. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

Figure 2

Hypoxia promotes Charcot-Leyden crystal formation but not degranulation.

(a) Representative images of CLC formation in eosinophils from an asthmatic donor cultured under hypoxia for 48 hrs; arrow indicates CLC; scale bar indicates a length of 10 μ M. (b) Eosinophils cultured under normoxia or hypoxia were manually scored for the number of CLCs visible in 5 random fields of view (x40 objective) per slide. AU Arbitrary Units; 1 \leq 25 CLCs; 2 = 25-50 CLCs; 3 = 50+ CLCs. Data are mean from (n) = 4 independent experiments, each performed in triplicate. (c) Eosinophil actin polymerisation was assessed 4 min post-stimulation with eotaxin (100 ng/ml) following culture under normoxia (N) or hypoxia (H) for 1 hr, relative to vehicle-treated (control) or freshly isolated eosinophils; data represent the mean \pm SEM of (n) = 4 independent experiments. (d) EDN release into the supernatant was assessed by ELISA from healthy control eosinophils following 24 hrs culture with IL-5 (10 ng/ml) or vehicle under N or H. Data represent the mean \pm SEM of (n) = 3 independent experiments. */# p <0.05; ** p <0.01; *** p <0.001; ****/##### p <0.0001 (* relative to N control; # relative to N equivalent).

Figure 3

Hypoxia promotes eosinophil survival, which correlates with reduced uptake by macrophages.

Eosinophil apoptosis was assessed by flow cytometry (a) or morphology (b) following 24 hrs culture under N or H, with or without IL-5 (10 ng/ml). Data represent mean \pm SEM from (n) = 8 independent experiments. Representative flow plots and images are shown in the left-hand panels. (c) Eosinophils aged under N or H for 24 hrs were co-cultured with macrophages for 1 hr under N or H and the degree of efferocytosis quantified.

Representative well images are shown in the left-hand panel. Data represent the mean \pm SD from (n) = 2 independent experiments. **** $p < 0.0001$ relative to N control.

Figure 4

Hypoxia-mediated eosinophil survival is regulated by NF- κ B and PI3-kinase.

Eosinophil apoptosis was assessed after 24 hrs culture under normoxia or hypoxia in the presence or absence of (a) the NF- κ B inhibitor GSK657311A (1 μ M-30 μ M), or (b) the Class 1A PI3-kinase inhibitor 987740A (1 μ M-10 μ M) a pan-PI3K inhibitor LY294002 (LY; 10 μ M) or the JNK inhibitor SP600125 (SP6; 10 μ M). Data represent the mean \pm SEM of data from (n) = 4 independent experiments. ** $p < 0.01$, *** $p < 0.001$ relative to N control; $\neq p < 0.05$, $\neq \neq p < 0.01$ relative to N equivalent; # $p < 0.05$, ### $p < 0.001$ relative to H control.

Figure 5

Concentration-dependent induction of eosinophil apoptosis by GCs is attenuated under hypoxia.

(a) Eosinophil apoptosis, measured by flow cytometry, was assessed at 24 hrs following cultured under N or H in the presence or absence of dexamethasone (1 nM-1 μ M), RU486 (10 μ M), IL-5 (10 ng/ml). (b) Percentage of apoptotic eosinophils at 24 hrs following N or H cultures with dexamethasone (Dex: 1 μ M), fluticasone furoate (FF, 100 nM), IL-5 (10 ng/ml) or vehicle control. (c) Representative flow cytometry of AnnexinV/PI stained eosinophils and cytocentrifuge images (x40) following a 24 hr culture with or without Dex under N or H. (d) Representative time course of eosinophil apoptosis following culture

with Dex (1 μ M), IL-5 (10 ng/ml) or vehicle under N or H over 120 hrs. (e) Percentage of eosinophil apoptosis when cultured under N or H with Dex (1 μ M) and hypoxia mimetics, DFO (1 mM) and DMOG (100 μ M) for 24 hrs. All data (except (d)) represent mean \pm SEM of (n) = 4 independent experiments; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ relative to N control; $\neq p < 0.01$, $\neq\neq p < 0.0001$ relative to the N equivalents.

Figure 6

GCS promote eosinophil apoptosis via a GR-transrepressive and oxygen-dependent mechanism.

(a) Levels of Gilz (steroid-regulated) and GLUT1 (hypoxia-regulated) mRNA expression relative to I κ B levels were examined in eosinophils cultured under N or H for 12 hour in the presence of Dex. All transcript levels were normalised to housekeeping control beta-2 microglobulin. (b) Eosinophils were treated with increasing concentrations of the GR modulator GRT10 under N or H for 24 hrs and assessed for the degree of apoptosis by flow cytometry. Data represent mean \pm SEM of (n) = 4 independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to N control.

Figure S1

Hypoxia upregulates IL-8 and MIP1 β release from asthmatic and healthy donors, whilst the secretion of MCP-1, RANTES, MIP1 α , eotaxin and Macrophage-derived chemokine (MDC) are unaltered.

Eosinophils isolated from two healthy controls and one atopic donor were cultured for 12 hrs under N or H and the amount of cytokine or chemokine released into the supernatants

assessed by ELISA. No factors were detected in supernatants derived from freshly isolated eosinophils (data not shown).

Figure S2

Hypoxia potentiates eotaxin-induced actin polymerization.

Eosinophils were cultured for 1 hr under N or H and then treated with 100 ng/ml eotaxin or vehicle and fixed with 4% PFA, permeabilised and stained for actin with NBD-buffer and compared to freshly isolated cells. The degree of actin polymerisation was assessed by flow cytometry with the degree of relative F-actin calculated as the ratio of the mean channel fluorescence between normoxic and hypoxic eotaxin-stimulated and non-stimulated cells. Data represent mean \pm SEM from (n) = 4 independent experiments.

Figure S3

Phagocytosis of apoptotic neutrophils using light microscopy and quantitation of MDM ϕ phagocytosis of apoptotic neutrophils by flow cytometry.

(a) Human M-CSF differentiated macrophages were co-incubated with apoptotic neutrophils for 1 hr under N or H before being washed and fixed with 2.5% glutaraldehyde and stained for MPO. Representative images are shown. (b) Phagocytosis was quantified by counting the number of macrophages (Macs) that had engulfed one or more neutrophil from duplicate wells (across five fields of view) using light microscopy. (c) Phagocytosis was additionally assessed using flow cytometry by co-incubating macrophages with CMFDA-labelled apoptotic neutrophils for 1 hr under N or H (ingested CMFDA-labelled apoptotic neutrophils were detected as FL-1 positive).

Representative density plots of the percentage of macrophages able to phagocytose CMFDA-labelled apoptotic neutrophils under N or H are shown. (d) To compare the effects of hypoxia mimetics, macrophages were pre-treated with DMOG (0.1–1 mM) or vehicle for 30 min before incubation under N with CMFDA-labelled apoptotic neutrophils for 1 hr before being assessed by flow cytometry. Data represent mean \pm SEM of data from (n) = 8 (b) or (n) = 3 (D) independent experiments, each conducted in duplicate. # $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to N control.

Figure S4

GCs promote neutrophil survival via a GR-transrepressive and oxygen-dependent mechanism.

(a) Purified human neutrophils (PMN) or eosinophils (Eos) were treated with GR modulator GRT10 (100 μ M - 100 pM) or vehicle control and cultured for 20 hours under N or H and assessed for apoptosis by flow cytometry. (b) Purified human neutrophils and eosinophils treated with dexamethasone or GRT10 (1 nM or 1 μ M) or vehicle control were cultured for 6 hours under N before RNA was harvested and expression of *Gilz* assessed by qPCR. Data in (a) represent mean \pm SEM of data from (n) = 4 independent experiments, each conducted in duplicate; * $p < 0.05$. Data in (b) represent mean \pm SD of (n) = 2 independent experiments, each conducted in triplicate.

Figure S5

Effect of hypoxia and dexamthasone on GC-mediated transrepression of IL-8 in eosinophils.

Eosinophils from healthy non-atopic (A) or atopic donors (B) were pre-incubated at 2×10^6 /ml at 37°C for 8 hours in 5% CO₂ in a normoxic or hypoxic environment before being pre-treated with Dex (1 µM) or vehicle for 1 hour before the further addition of TNFα (10 ng/ml) or media for a further 3 hours under the same conditions. The amount of IL-8 released was assessed by ELISA and expressed in (pg/ml). No IL-8 was detected from freshly isolated eosinophils (data not shown). Data represent mean \pm SEM of data from (n) = 3 (healthy non-atopic donors, A) or (n) = 7 (atopic donors, B) independent experiments.

* $P < 0.05$ relative to the normoxic equivalent.

Footnotes

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Abbreviations: AnV, Annexin V; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; CLC, Charcot-Leydon crystal; DFO, Desferrioxamine; DMOG, Dimethyloxaloyllylglycine; EDN, Eosinophil-derived neurotoxin; Eos, Eosinophil; F-actin, Filamentous actin; FF, Fluticasone furoate; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GC, Glucocorticosteroid; GILZ, Glucocorticoid-inducible leucine zipper; GLUT1, Glucose transporter 1; GR, Glucocorticoid receptor; H, Hypoxia (0.8% oxygen); HIF, Hypoxia inducible factor; MDM ϕ , Monocyte-derived macrophage; MPO, Myeloperoxidase; N, Normoxia (21% oxygen); PBS+, PI, Propidium iodide; PMN, Polymorphonuclear cell; rhIL-8, Recombinant human interleukin-8; SNAP, Soluble NSF Attachment Protein; SNARE, Soluble NSF Attachment Protein Receptor; VEGF, Vascular endothelial growth factor.

Acknowledgments: This work was funded by a BBSRC Industrial CASE Partnership Grant and the NIHR Cambridge Biomedical Research Centre. CAF held a MRC Clinical Research Training Fellowship. We are grateful for the technical support of Ms Ros Simmonds and staff of Cambridge University Hospitals NHS Foundation Trust.

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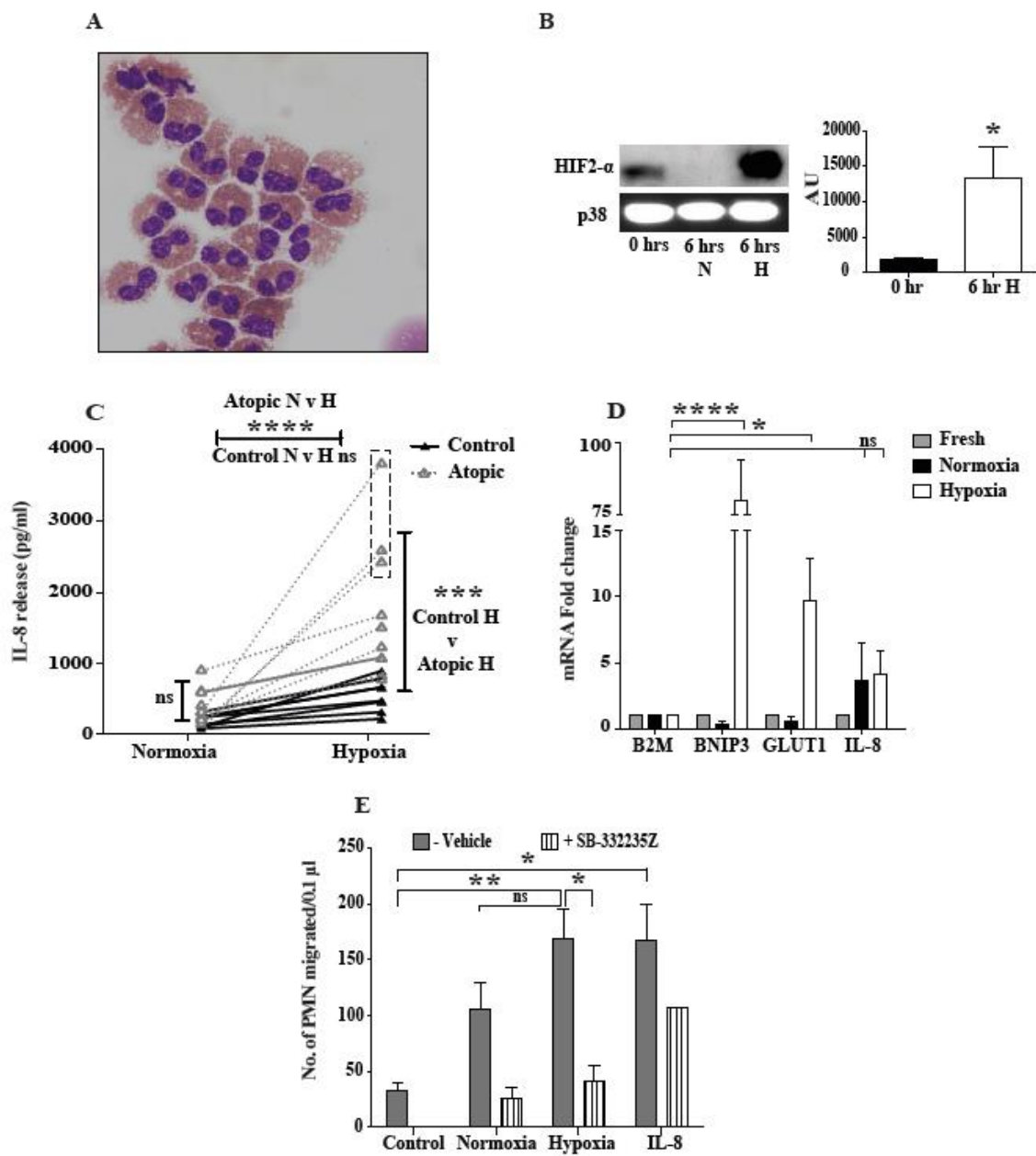


Figure 1

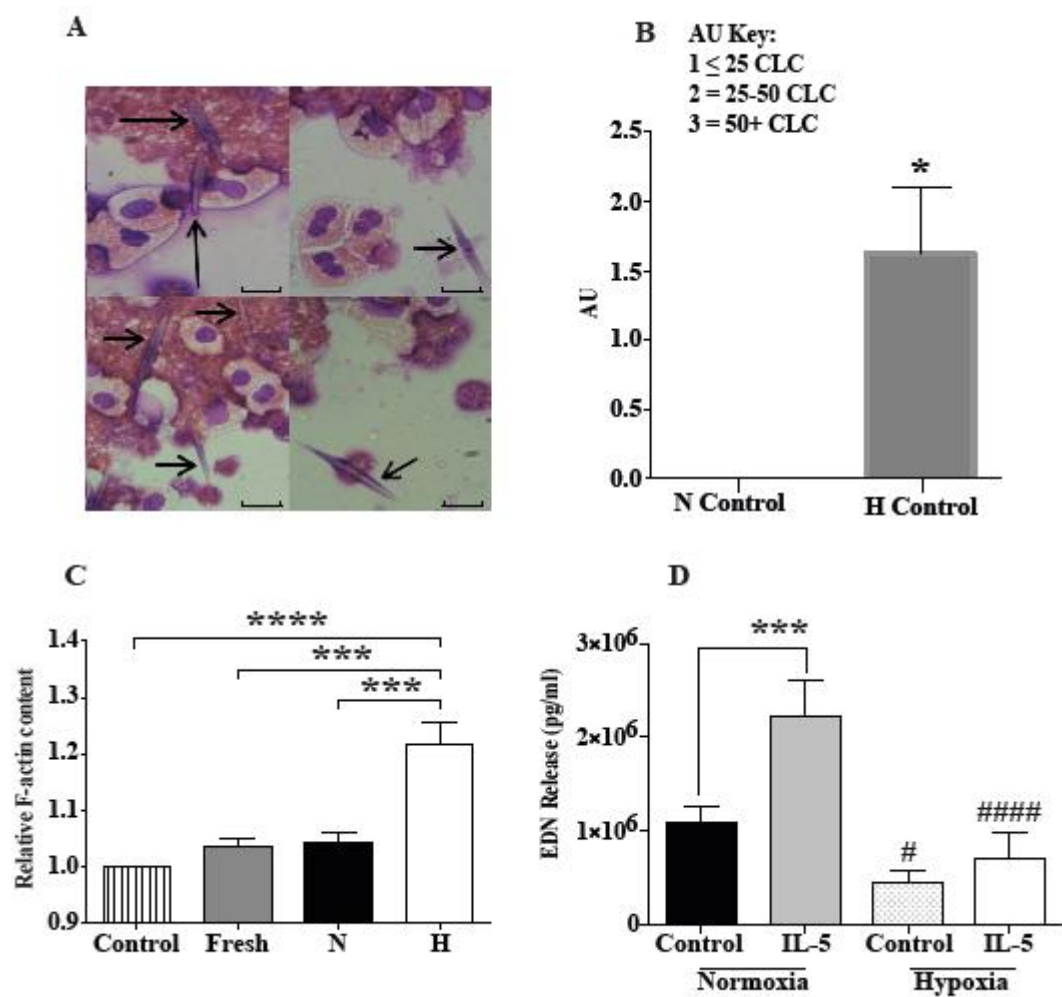


Figure 2

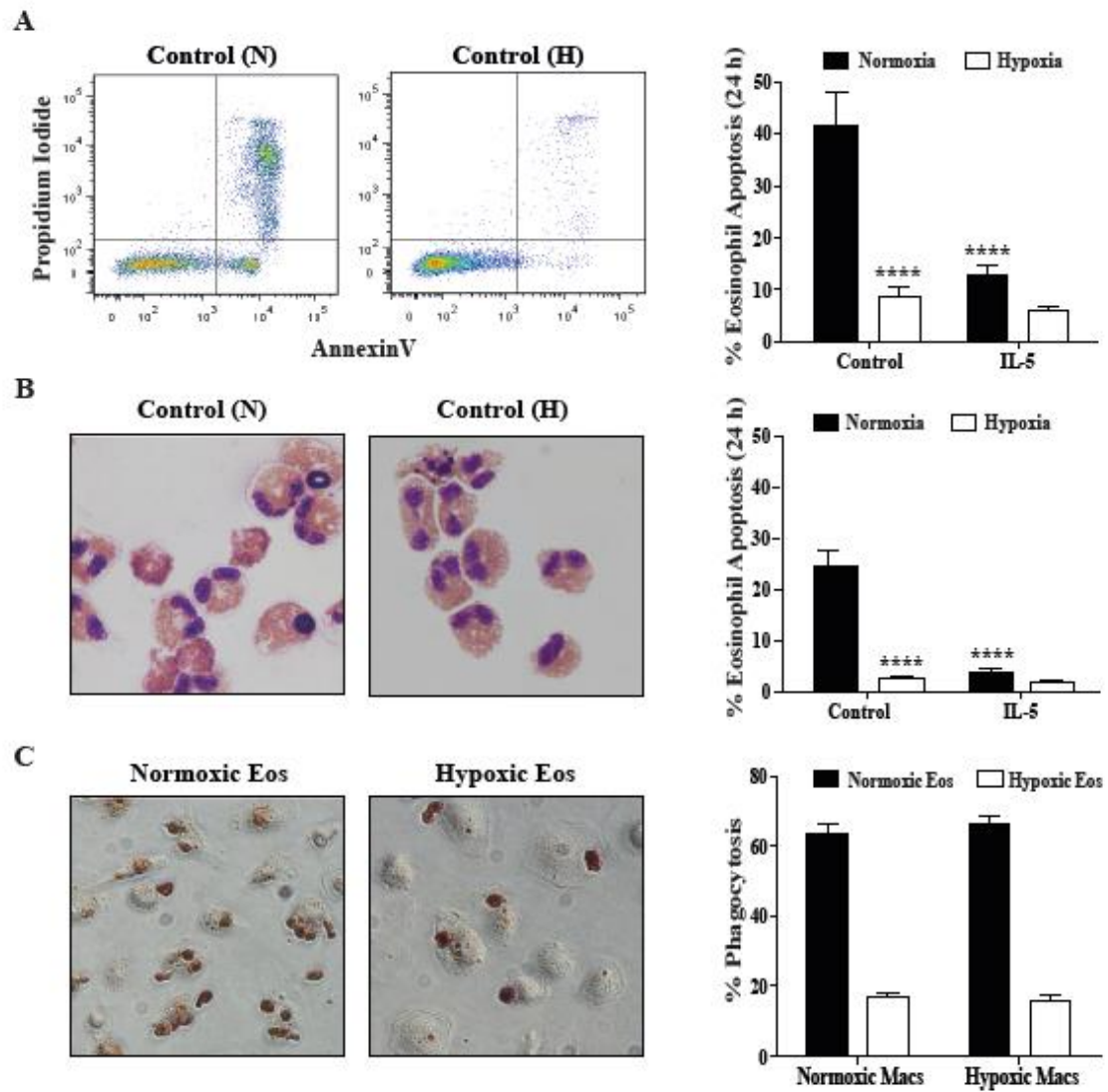


Figure 3

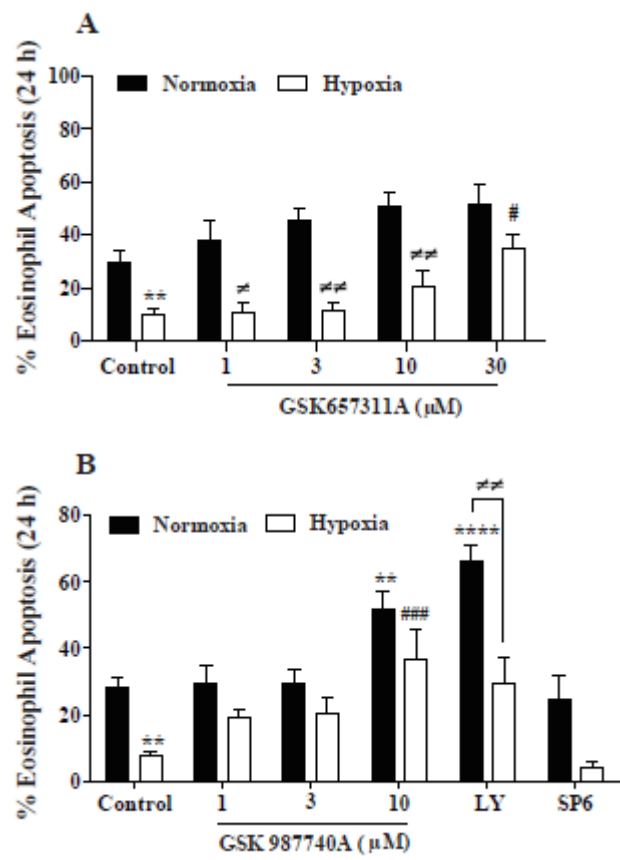


Figure 4

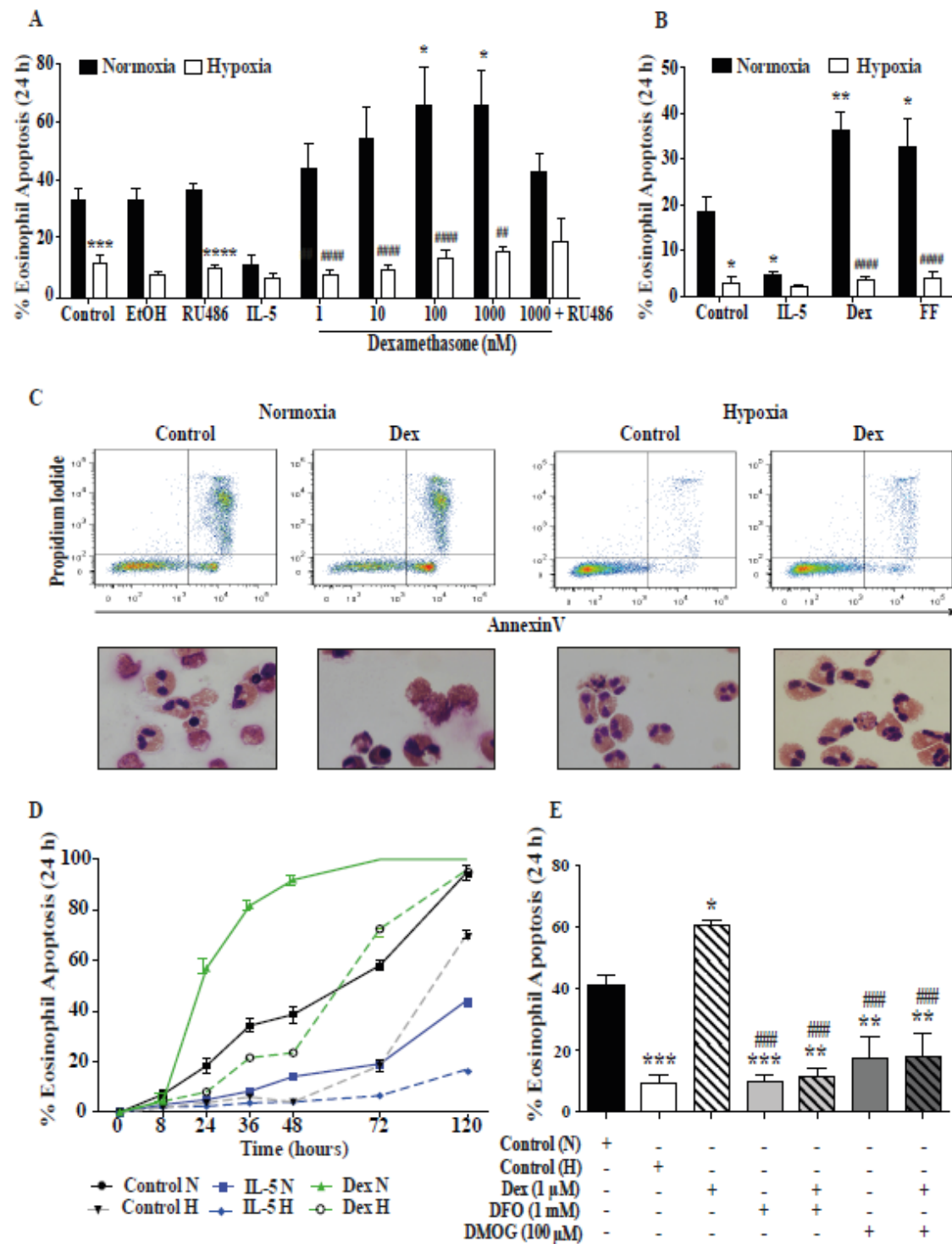


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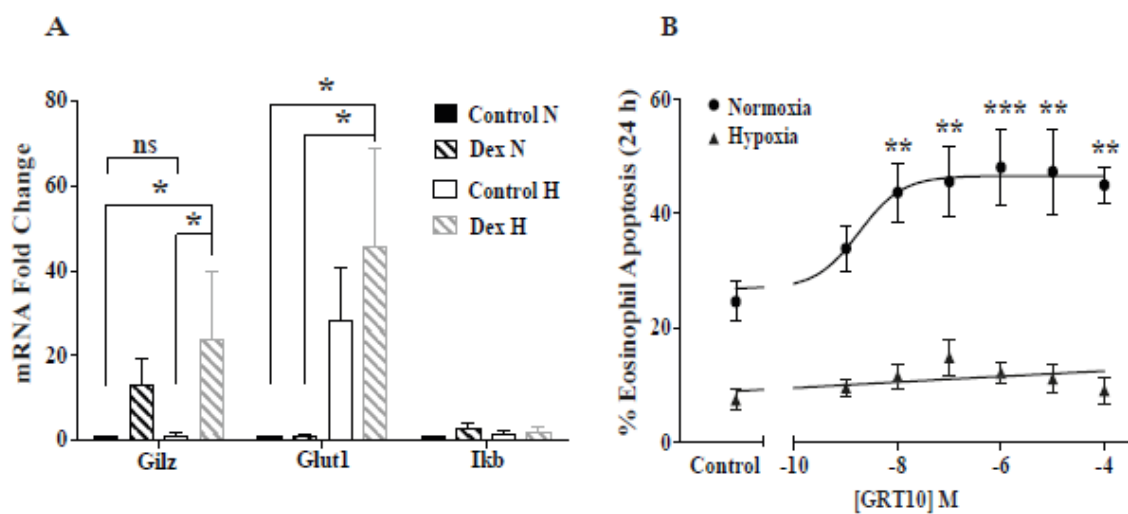


Figure 6

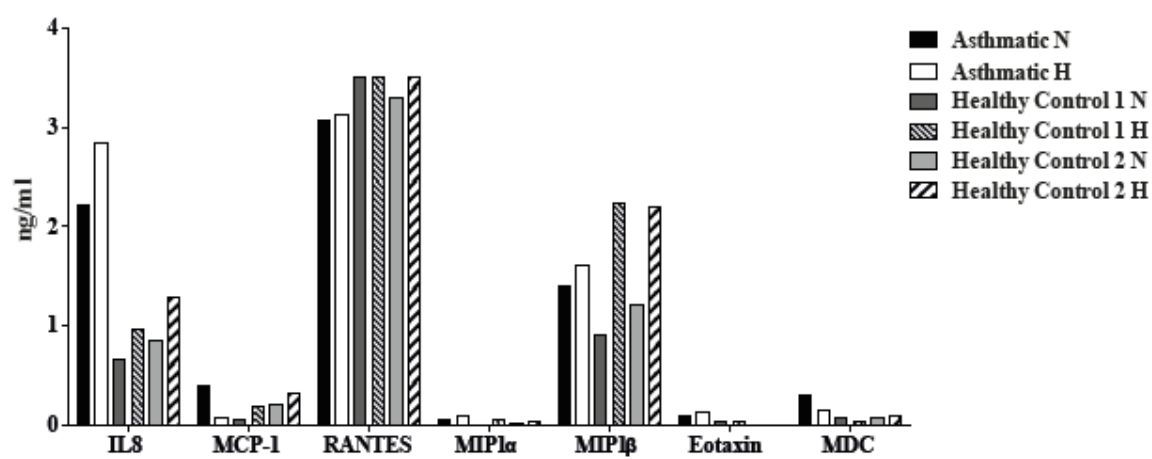


Figure S1

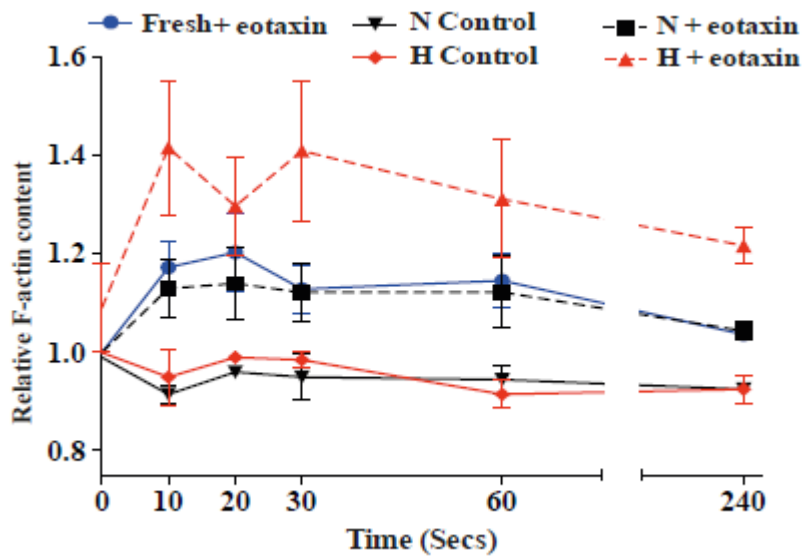


Figure S2

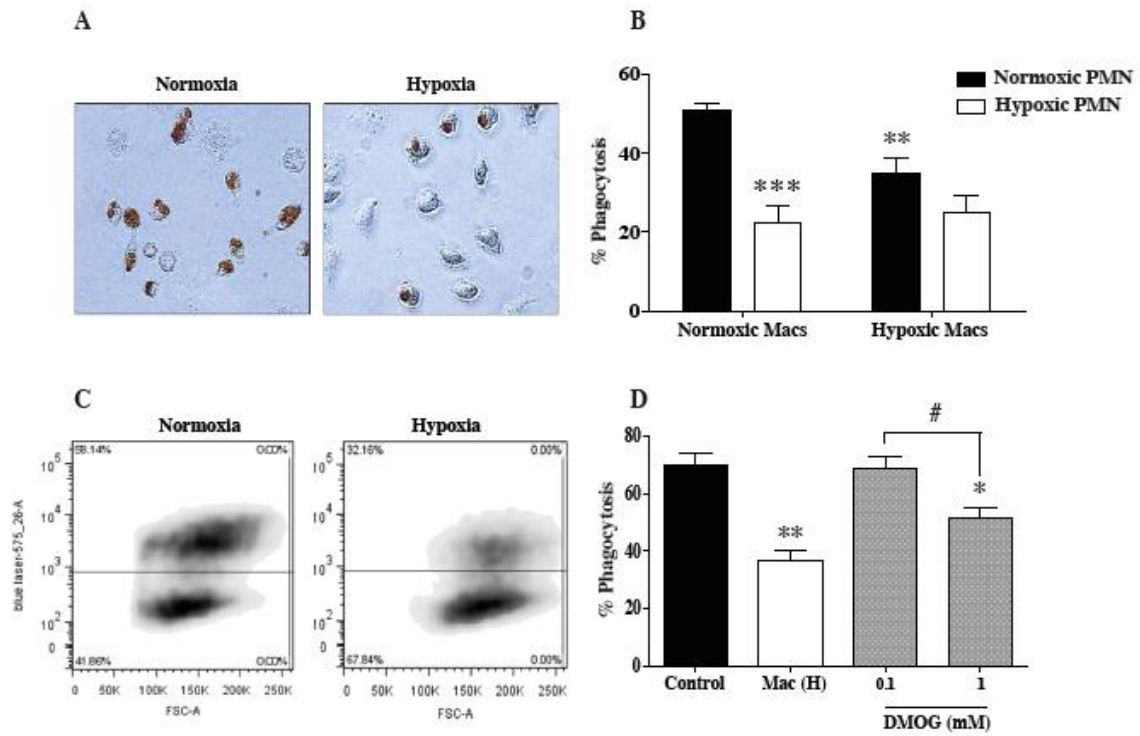


Figure S3

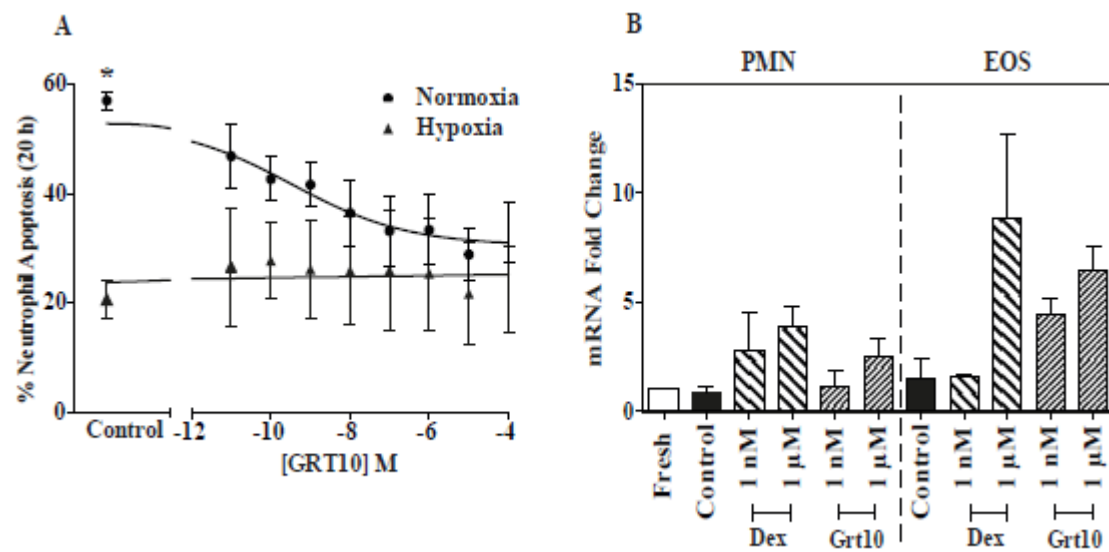


Figure S4

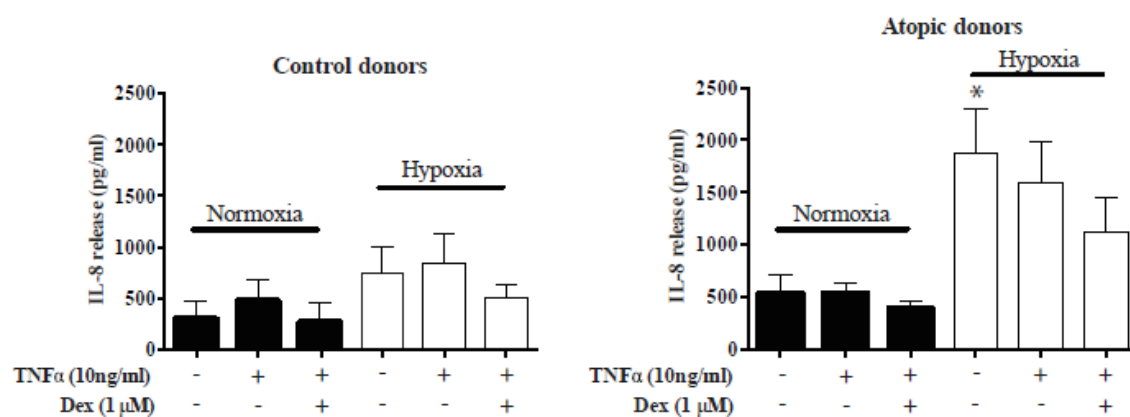


Figure S5